On the heterogeneity of the mitochondrial C_{27}-steroid 26-hydroxylase system

Jan Gustafsson

Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

Abstract Mitochondrial 26-hydroxylation of exogenous cholesterol, endogenous cholesterol, and 5β-cholestane-3α,7α,12α-triol was studied. 26-Hydroxylation of endogenous cholesterol was measured by mass fragmentography. NADPH and isocitrate stimulated 26-hydroxylation of endogenous as well as exogenous cholesterol. 26-Hydroxylation of endogenous cholesterol was linear with time for 15 min, whereas that of exogenous cholesterol was linear with time for at least 40 min. This finding indicates that the fractions of exogenous and endogenous cholesterol that were 26-hydroxylated did not equilibrate. Mg^{2+} stimulated isocitrate- and NADPH-dependent 26-hydroxylation of exogenous cholesterol but inhibited in the case of endogenous cholesterol. Ca^{2+} stimulated NADPH-dependent and inhibited isocitrate-dependent 26-hydroxylation of both exogenous and endogenous cholesterol. It is suggested that the differing effect of Mg^{2+} on the 26-hydroxylation of exogenous and endogenous cholesterol is related to transfer of the steroid to the enzyme. Isocitrate- and NADPH-dependent 26-hydroxylation of exogenous 5β-cholestane-3α,7α,12α-triol differed from that of exogenous cholesterol in response to Mg^{2+} and Ca^{2+}. 26-Hydroxylation of 5β-cholestane-3α,7α,12α-triol was stimulated by Mg^{2+} in low concentrations but inhibited by Mg^{2+} and Ca^{2+} in high concentrations. Mg^{2+} had the same influence on the 26-hydroxylation of three dioxygenated C_{27}-steroids known to be intermediates in bile acid biosynthesis. The results are not only compatible with heterogeneity of the mitochondrial 26-hydroxylase system but also with differences in the transport of cholesterol and 5β-cholestane-3α,7α,12α-triol to the enzyme. The finding of a differing effect of Mg^{2+} on 26-hydroxylation of exogenous and endogenous cholesterol seems to favor differences in transport rather than heterogeneity of the 26-hydroxylase as an explanation of the results.

Supplementary key words mitochondrial 26-hydroxylation · exogenous cholesterol · endogenous cholesterol · 5β-cholestane-3α,7α,12α-triol · steroid transfer

In the main pathways for the conversion of cholesterol into bile acids in the rat, 26-hydroxylation of the C_{27}-steroid side chain is a necessary step (1). The reaction can be catalyzed by two different enzyme systems with different subcellular localizations and different properties (2). The 26-hydroxylase located in the microsomal fraction of rat liver homogenate shows highest activity with 5β-cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol as substrates but it is not active on cholesterol (2).

The mitochondrial fraction 26-hydroxylates every tested intermediate in the biosynthesis of bile acids (2), but the preferred substrates are 7α-hydroxy-4-cholesten-3-one and 5β-cholestane-3α,7α,12α-triol, with cholesterol reacting at a considerably slower rate. In the main pathways for the formation of chenodeoxycholic and cholic acids, the major substrates for mitochondrial 26-hydroxylation are supposed to be those given above for microsomal 26-hydroxylation. The relative importance of microsomal and mitochondrial 26-hydroxylation has not been definitely established, but evidence has been obtained to indicate a major role for the former (3).

Alternative pathways for chenodeoxycholic acid formation involving mitochondrial 26-hydroxylation of cholesterol or 5-cholestene-3β,7α-diol have been described (4, 5). The contribution of these pathways to total chenodeoxycholic acid formation may be small under normal conditions but may become important under a condition such as biliary obstruction, which is characterized by a shift from cholic acid synthesis to chenodeoxycholic acid synthesis (6).

In a recent report from this laboratory on mitochondrial 26-hydroxylation of exogenous cholesterol it was found that the 26-hydroxylase system was a monooxygenase requiring NADPH, probably generated intramitochondrially (7). The system was associated with the inner mitochondrial membrane and was inhibited by carbon monoxide, while the addition of relatively high concentrations of Mg^{2+} and Ca^{2+} stimulated the reaction markedly. Cholesterol was chosen as the substrate in that study because its side chain is not 26-hydroxylated by the microsomes that always contaminate mitochondrial preparations to some extent.

Abbreviations: 25-hydroxycholesterol, 5-cholestene-3β,25-diol; 26-hydroxycholesterol, 5-cholestene-3β,26-diol.

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In an independent study, Taniguchi, Hoshita, and Okuda (8) reported on the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol by the mitochondrial fraction of rat liver homogenate. The properties of this reaction were very similar to those of the 26-hydroxylation of cholesterol. Taniguchi et al. (8) did not study the effects of Mg^{2+} and Ca^{2+} in any detail.

Since preliminary experiments in this laboratory indicated differences between the 26-hydroxylation of exogenous cholesterol and of 5β-cholestane-3α,7α,12α-triol in response to these ions, it appeared of interest to examine this question further. In previous work, the assumption was made that exogenous cholesterol mixed with endogenous mitochondrial cholesterol (2, 7). Since methods have recently been developed in this laboratory for studying simultaneously the metabolism of exogenous and endogenous cholesterol (9), it was also possible to make a comparison between the mitochondrial 26-hydroxylations of these compounds.

METHODS

Materials

[4-^{14}C]Cholesterol (60 μCi/μmole) was obtained from the Radiochemical Centre, Amersham, England. Prior to use, the material was purified by chromatography on a column of aluminum oxide, grade III (Woelm, Eschwege, Germany). The labeled cholesterol was shown by mass spectrometry to contain about a 90 atom percent excess of ^{14}C. 5β-[7β-^{3}H]Cholestane-3α,7α,12α-triol (7 μCi/μmole) was synthesized as described previously (10). (25R)26-Hydroxycholesterol was prepared from kryptogenin as described by Scheer, Thompson, and Mosettig (11). 5β-Cholestane-3α,7α,12α,26-tetrol was prepared as described previously (2). Cofactors were obtained from Sigma Chemical Co., St. Louis, Mo.

Preparation of mitochondrial fraction

Male rats of the Sprague-Dawley strain weighing about 200 g were used. The mitochondrial fraction was prepared according to Sottocasa et al. (12). The average protein content was 6–8 mg per ml of mitochondrial fraction when determined according to Lowry et al. (13). The average content of free cholesterol was 20–30 μg per ml of mitochondrial fraction as determined by mass fragmentography. Esterified cholesterol amounted to 2–6 μg per ml.

With the technique used to prepare the mitochondrial fraction (12), the degree of microsomal contamination should be quite low. When microsomal 12α-hydroxylase activity was analyzed as an indicator of microsomal contamination, there was no more than 2–4% contamination of the mitochondrial fraction. A similar figure was calculated from the extent of 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol, the main microsomal hydroxylation of this substrate (10).

Procedures for incubations and analyses

In standard incubations [4-^{14}C]cholesterol, 20 or 100 μg dissolved in 50 μl of acetone, or 5β-[7β-^{3}H]-cholestane-3α,7α,12α-triol, 100 μg in 50 μl of acetone, was incubated with 1.0 ml of mitochondrial fraction and 3 μmoles of NADPH or 4.6 μmoles of isocitrate in a total volume of 3 ml. When 26-hydroxylation of endogenous cholesterol was studied, 20 μg of [4-^{14}C]cholesterol was added, although the amount required to saturate the system is about 80 μg (7). The lower amount was used for two reasons: it corresponded to the amount of endogenous cholesterol, and it offered the best conditions for measurements of hydroxylation of endogenous cholesterol. Blank incubations were performed using only buffer or boiled mitochondrial fraction.

Incubations were terminated and extracted as described previously (2, 7). The residues of the extracts were subjected to thin-layer chromatography using benzene–ethyl acetate 1:1 (v/v) in the case of cholesterol (7), and system S 7 (benzene–isopropanol–acetic acid 30:10:1, v/v/v), in the case of 5β-cholestane-3α,7α,12α-triol (14). The thin-layer plates were analyzed for radioactivity using a thin-layer scanner (Berthold, Karlsruhe, Germany). The chromatographic zones containing 26-hydroxycholesterol and 5β-cholestane-3α,7α,12α,26-tetrol were eluted with methanol and converted into trimethylsilyl ethers. Part of the trimethylsilyl ethers was subjected to radio–gas–liquid chromatography (2).

In the case of incubations concerned with analyses of the 26-hydroxylation of endogenous cholesterol, the cholesterol zone and part of the 26-hydroxycholesterol zone from the thin-layer chromatograms were analyzed by combined gas–liquid chromatography–mass spectrometry using an LKB 9000 instrument equipped with a MID-unit (multiple ion detector). The experimental conditions were as described by Björkhem and Danielsson (15). The first channel of the MID-unit was focused on the ion at *m/e* 456 (M − 90 of the trimethylsilyl ether of unlabeled 26-hydroxycholesterol) and the second at *m/e* 458 (M − 90 of the trimethylsilyl ether of [4-^{14}C]26-hydroxycholesterol). The third channel was also focused on *m/e* 458 (M of the trimethylsilyl ether of unlabeled cholesterol). The fourth

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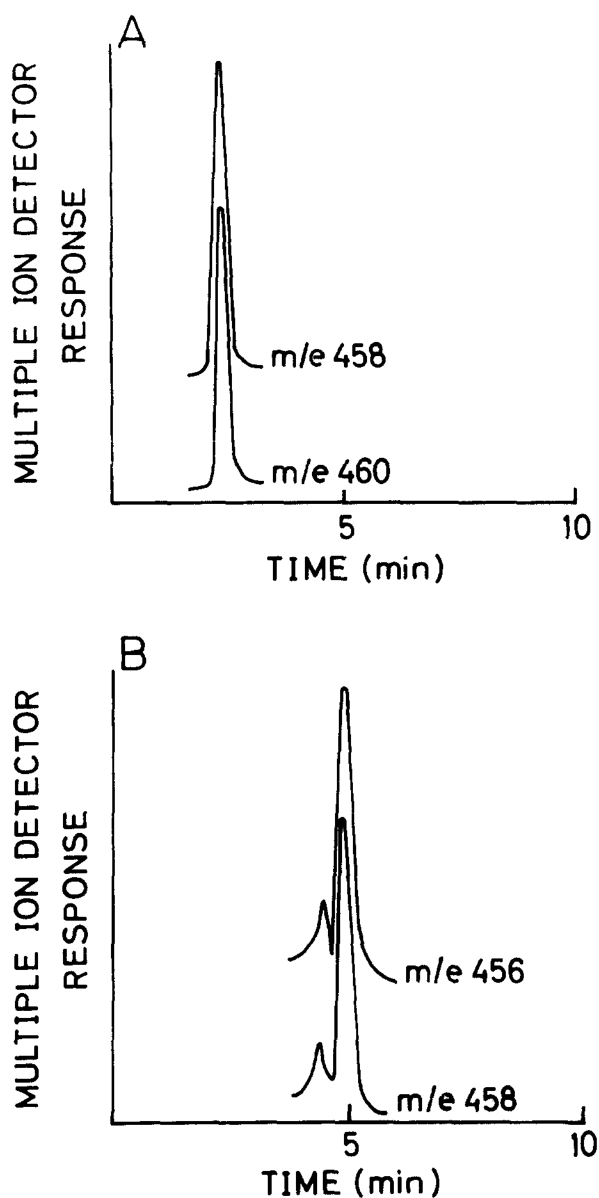

Fig. 1. Mass-fragmentographic analysis with a 1.5% SE-30 column of material from an incubation of mitochondrial fraction with [4-^{14}C]cholesterol, 20 μg (50 nmoles), and isocitrate, 4.6 μmoles for 40 min. (A) shows a mass-fragmentographic representation of *m/e* 458 and *m/e* 460 for the trimethylsilyl ether of material obtained from the thin-layer chromatographic zone corresponding to cholesterol. The degree of amplification was 90× for both channels. (B) shows a mass-fragmentographic representation of *m/e* 456 and *m/e* 458 for the trimethylsilyl ether of material obtained from the thin-layer chromatographic zone corresponding to 26-hydroxycholesterol. The peak at 4.5 min corresponds to 25-hydroxycholesterol and the peak at 5.0 min corresponds to 26-hydroxycholesterol. The degree of amplification was 300× for both channels.

channel was focused on *m/e* 460 (M of the trimethylsilyl ether of labeled cholesterol). The degree of amplification was the same for channels 1 and 2, 300× or 900×, and for channels 3 and 4, 30× or 90×. **Fig. 1** shows a mass-fragmentographic analysis of an incubation with 20 μg of [4-^{14}C]cholesterol. The zero time incubations contained less than 0.02 μg of labeled and unlabeled 26-hydroxycholesterol.

Calculations

The amounts of [4-^{14}C]26-hydroxycholesterol and 5β-[7β-^{3}H]cholestane-3α,7α,12α,26-tetrol formed were calculated from the percent conversion measured by thin-layer chromatography and radio–gas–liquid chromatography (2). In incubations with [4-^{14}C]cholesterol, the amounts of endogenous cholesterol and endogenously formed 26-hydroxycholesterol were calculated as described by Björkhem and Karlmar (9). The equation presented below shows calculations for 26-hydroxycholesterol. Endogenous cholesterol was calculated in an analogous manner.

$$\frac{I_{456}}{I_{458}} = \frac{\dfrac{I_{456},{}^{14}C}{I_{456},{}^{14}C + I_{458},{}^{14}C} W_{ex} + \dfrac{I_{456},{}^{12}C}{I_{456},{}^{12}C + I_{458},{}^{12}C} W_{en}}{\dfrac{I_{458},{}^{14}C}{I_{456},{}^{14}C + I_{458},{}^{14}C} W_{ex} + \dfrac{I_{458},{}^{12}C}{I_{456},{}^{12}C + I_{458},{}^{12}C} W_{en}}$$

I_{456} and I_{458}: Tracings at *m/e* 456 and 458 obtained from trimethylsilyl ether of the thin-layer chromatographic zone corresponding to 26-hydroxycholesterol.

I_{456}, ^{12}C and I_{458}, ^{12}C: Tracings at *m/e* 456 and 458 obtained from trimethylsilyl ether of unlabeled 26-hydroxycholesterol.

I_{456}, ^{14}C and I_{458}, ^{14}C: Tracings at *m/e* 456 and 458 obtained from trimethylsilyl ether of [4-^{14}C]26-hydroxycholesterol (cf. text).

W_{ex}: Weight of [4-^{14}C]26-hydroxycholesterol.

W_{en}: Weight of unlabeled (endogenous) 26-hydroxycholesterol.

As no [4-^{14}C]26-hydroxycholesterol was available, calculations concerned with this compound made use of the ratio *m/e* 458: *m/e* 460 that was determined for the trimethylsilyl ether of [4-^{14}C]cholesterol in place of the ratio *m/e* 456: *m/e* 458. The validity of this approximation is strengthened by the finding that the ratio *m/e* 456: *m/e* 458 for the trimethylsilyl ether of unlabeled 26-hydroxycholesterol was essentially the same as the ratio *m/e* 458: *m/e* 460 for the trimethylsilyl ether of unlabeled cholesterol.

RESULTS

After incubation of mitochondrial fraction with 20 μg of [4-^{14}C]cholesterol in the presence of NADPH and 10 mM Mg^{2+}, the formation of 26-hydroxycholesterol from endogenous cholesterol was linear with time up to about 15 min and then leveled off (**Fig. 2**). Omission of NADPH from the

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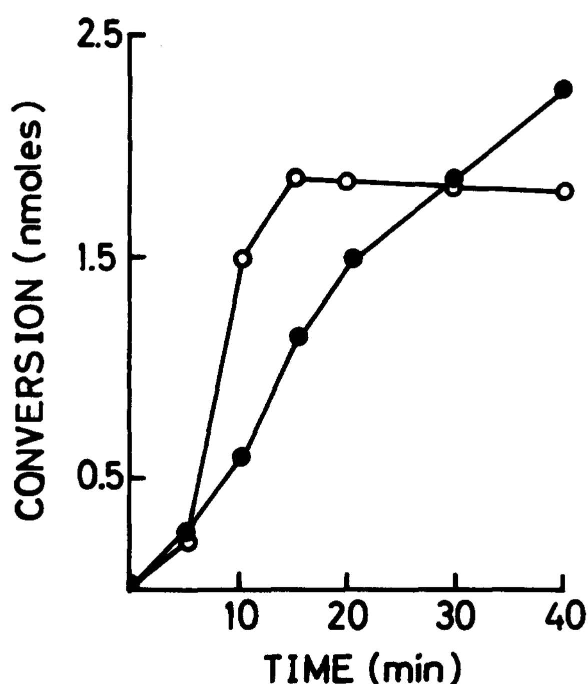

Fig. 2. Effect of time on mitochondrial, NADPH-dependent 26-hydroxylation of [4-^{14}C]cholesterol, 20 μg (●), and endogenous cholesterol (○). Standard incubation conditions were used with Mg^{2+}, added to 10 mM concentration.

system decreased 26-hydroxylation of endogenous cholesterol by about 50% (**Table 1**). When NADPH was replaced by isocitrate, the rate of reaction was twice that observed with NADPH (Table 1).

In agreement with previous findings (7) the rate of 26-hydroxylation of exogenous cholesterol in the presence of NADPH and 10 mM Mg^{2+} was linear with time up to at least 40 min (Fig. 2). Either NADPH or isocitrate was required in this reaction.

26-Hydroxylation of endogenous cholesterol in the presence of NADPH or isocitrate differed from that of exogenous cholesterol in its response to Mg^{2+}. Whereas 26-hydroxylation of exogenous cholesterol below or at substrate saturation was stimulated by increasing concentrations of Mg^{2+}, 26-hydroxylation of endogenous cholesterol was inhibited by Mg^{2+} in concentrations of 3 mM or higher (Table 1). When Mg^{2+} was replaced by 10 mM Ca^{2+} in incubations with NADPH, 26-hydroxylation of endogenous cholesterol was stimulated two- to threefold (Table 1), and that of exogenous cholesterol was also stimulated. In incubations in which isocitrate was used instead of NADPH, 10 mM Ca^{2+} inhibited or had no effect on the 26-hydroxylation of endogenous and exogenous cholesterol, whether or not exogenous cholesterol was added to give substrate saturation.

Initially the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol was studied in a system with 10 mM Mg^{2+} and NADPH, but a significant conversion was also obtained in the absence of cofactors. When NADPH was added in saturating amounts (3 μmoles), the rate of 26-hydroxylation was stimulated by only about 25%. When NADPH was replaced by isocitrate and the incubations were carried out in the absence of Mg^{2+}, the rate was three to five times above basal level, i.e., the rate of conversion obtained without cofactors (**Fig. 3** and Table 1). With isocitrate as cofactor, the reaction was linear with time for at least 40 min and was linear with the amount of mitochondrial protein up to at least 12 mg. About 40 μg of 5β-cholestane-3α,7α,12α-triol saturated the system (Fig. 3). The isocitrate- and NADPH-dependent 26-hydroxylation was stimulated twofold by 1 mM Mg^{2+} but was inhibited by concentrations higher than 3 mM. Ca^{2+} in 10 mM concentration inhibited the reaction in presence of both NADPH

TABLE 1. Effect of cofactors, Mg^{2+} and Ca^{2+} on 26-hydroxylation of endogenous cholesterol, exogenous cholesterol, and 5β-cholestane-3α,7α,12α-triol

Addition	Endogenous Cholesterol	Exogenous Cholesterol, 20 μg	Exogenous Cholesterol, 100 μg	5β-cholestane-3α,7α, 12α-triol, 100 μg
	Conversion into 26-hydroxylated products			
	pmoles/mg protein/min			
None	4.0	0.5	0.5	21.0
NADPH	8.7	2.3	4.0	27.5
Isocitrate	14.7	5.5	10.0	75.0
NADPH + Mg^{2+}	6.0	5.0	20.0	13.8
Isocitrate + Mg^{2+}	10.7	7.0	20.0	42.0
NADPH + Ca^{2+}	18.7	7.0	35.8	13.8
Isocitrate + Ca^{2+}	8.7	5.0	6.7	13.8

The amount of NADPH was 3 μmoles and of isocitrate 4.6 μmoles. The concentrations of Mg^{2+} and Ca^{2+} were 10 mM. In the calculations of the conversions per minute, it was taken into consideration that 26-hydroxylation of endogenous cholesterol was linear with time for only 15 min.

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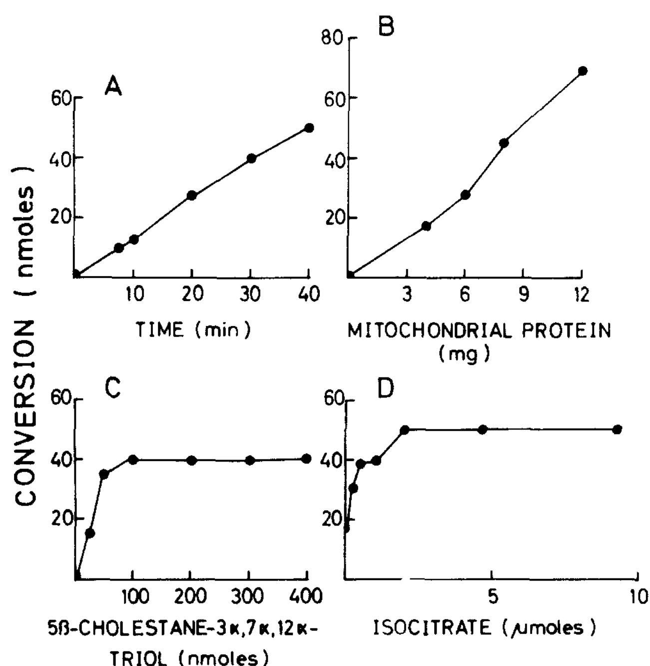

Fig. 3. Effect of time (A), mitochondrial protein concentration (B), substrate concentration (C) and isocitrate (D) on 26-hydroxylation of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol. Incubations were performed for 40 min except in (A), 8 mg of mitochondrial protein was used except in (B), 100 μg (250 nmoles) of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol were added except in (C) and 4.6 μmoles of isocitrate were used except in (D).

and isocitrate (Table 1). In all incubations with the 12α-triol the radioactivity present in the thin-layer chromatographic zone corresponding to 3α,7α,-12α-trihydroxy-5β-cholestanoic acid was less than 10% of that present as 5β-cholestane-3α,7α,12α,26-tetrol.

The influence of increasing concentrations of Mg^{2+} (1–10 mM) on 26-hydroxylation of various other C_{27}-steroids that are intermediates in bile acid biosynthesis was also tested. Parallel incubations with cholesterol and 5β-cholestane-3α,7α,12α-triol were carried out (**Fig. 4**). Mg^{2+} in concentrations of 1–3 mM stimulated the hydroxylations of 5-cholestene-3β,7α-diol, 7α-hydroxy-4-cholesten-3-one, and 5β-cholestane-3α,7α-diol whereas higher concentrations inhibited the reactions. Thus these substrates behaved towards Mg^{2+} in the same way as 5β-cholestane-3α,7α,12α-triol.

DISCUSSION

The ability of the mitochondrial fraction to catalyze 26-hydroxylation of various C_{27}-steroids in bile acid biosynthesis is well established (2). Recent studies in this laboratory (7) and by Taniguchi et al. (8) have shown that the 26-hydroxylase system(s) acting on cholesterol and 5β-cholestane-3α,7α,12α-triol is a carbon monoxide-sensitive monooxygenase located at the inner membrane or the inner membrane–matrix region. The NADPH required is probably generated intramitochondrially since the 26-hydroxylation of cholesterol as well as of 5β-cholestane-3α,7α,12α-triol is supported by isocitrate (7, 8).

The aim of the present investigation was to obtain information concerning the question whether or not the 26-hydroxylations of cholesterol and 5β-cholestane-3α,7α,12α-triol are catalyzed by the same enzyme. In such a study, it appeared important to assay separately the 26-hydroxylations of exogenous cholesterol and endogenous cholesterol. Such measurements should provide information concerning possible differences in transport of substrate to the enzyme. In this connection it may be pointed out that problems of compartmentation of endogenous cholesterol and of the extent of equilibration of endogenous and exogenous cholesterol have become increasingly evident in the case of the microsomal fraction of liver and the mitochondrial fraction of adrenal (9, 16).

In this laboratory a method was developed recently to measure simultaneously the metabolism of endogenous and of exogenous cholesterol (9). The method is based on gas–liquid chromatographic–mass-fragmentographic analysis of the amounts of unlabeled and ^{14}C-labeled molecules in cholesterol and its metabolic product(s) isolated after incubation of [4-^{14}C]cholesterol with a high atom percent excess of ^{14}C.

26-Hydroxylation of exogenous cholesterol was linear with time up to 40 min, whereas that of endogenous cholesterol leveled off after 10–15 min. This finding strongly indicates that exogenous cholesterol equilibrates only to a limited extent with the endogenous cholesterol that is 26-hydroxylated under the conditions employed. In studies on microsomal 7α-hydroxylation of cholesterol it was found that Tween 80 is superior to acetone in achieving equilibration of exogenous and endogenous cholesterol (16). In the present experiments, however, acetone was used as the solvent for cholesterol because the use of Tween 80 as solubilizing agent resulted in marked inhibition of 26-hydroxylation of both exogenous and endogenous cholesterol. Taniguchi et al. (8) found that various detergents also inhibited the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol.

The finding that both exogenous and endogenous cholesterol were 26-hydroxylated in spite of incomplete equilibration cannot be considered as evidence for more than one 26-hydroxylase for cholesterol. It

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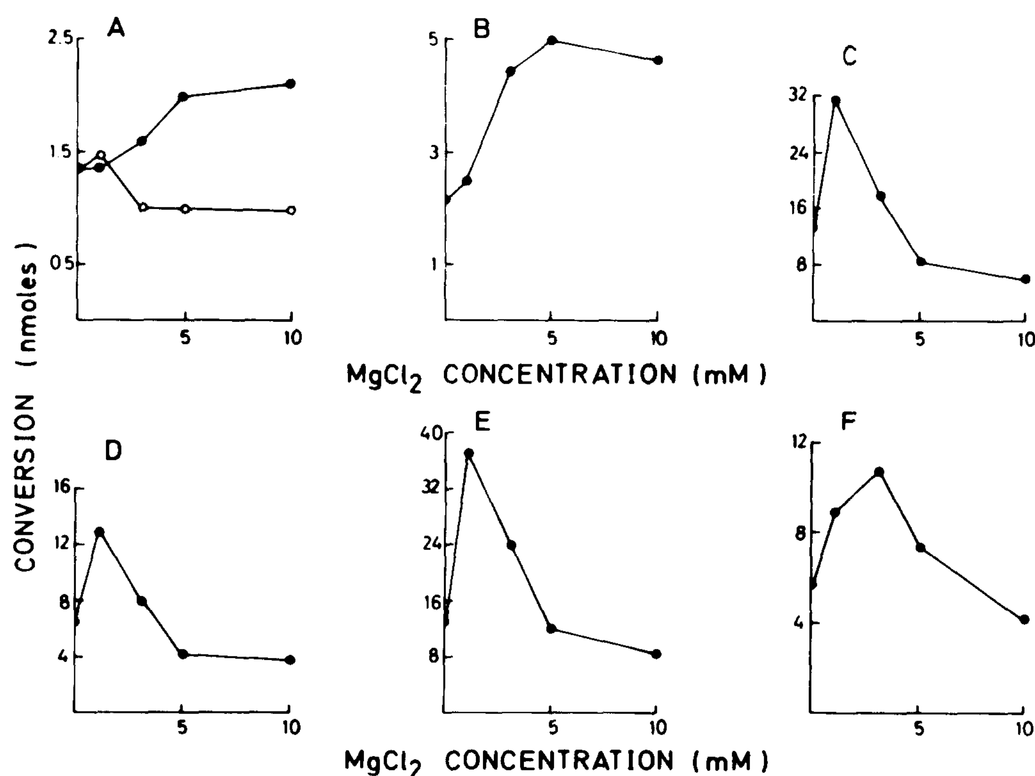

Fig. 4. Effect of Mg^{2+} on isocitrate-dependent 26-hydroxylation. In (A) 26-hydroxylation of endogenous cholesterol (○) and exogenous cholesterol (●), 20 μg (50 nmoles), was assayed; in (B) exogenous cholesterol, 100 μg (250 nmoles); in (C) 5β-cholestane-3α,7α,12α-triol, 100 μg (250 nmoles), in (D) 5-cholestene-3β,7α-diol 100 μg (250 nmoles); in (E) 7α-hydroxy-4-cholesten-3-one, 100 μg (250 nmoles); and in (F) 5β-cholestane-3α,7α-diol, 100 μg (250 nmoles).

is probable that endogenous cholesterol far from saturates the 26-hydroxylase system and that the system is thus able to hydroxylate exogenous cholesterol at the same time. The extent of hydroxylation of endogenous cholesterol was not influenced by the addition of cholesterol in saturating amounts or in amounts above saturation. This finding could indicate that endogenous cholesterol is more accessible to the enzyme and so is the preferred substrate.

It has been previously shown that 26-hydroxylation of exogenous cholesterol is accompanied by a 25-hydroxylation (7). Although not studied in detail, 26-hydroxylation of endogenous cholesterol was also accompanied by 25-hydroxylation and in a proportion similar to that observed for exogenous cholesterol (cf. Fig. 1).

Mg^{2+} in concentrations above 1 mM inhibited the 26-hydroxylation of endogenous cholesterol and stimulated that of exogenous cholesterol. There was no difference in response to Ca^{2+} between the reactions of exogenous and endogenous cholesterol. It seems reasonable that the differing effect of Mg^{2+} on the 26-hydroxylations of exogenous and endogenous cholesterol is related to transport of the two species of cholesterol to the enzyme.

Mg^{2+} in concentrations up to 3 mM stimulated 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. Higher concentrations resulted in either no effect or an inhibition. Ca^{2+} inhibited the reaction. Some other C_{27}-steroids known to be intermediates in the biosynthesis of bile acids responded to increasing additions of Mg^{2+} in the same way as 5β-cholestane-3α,7α,12α-triol. The differences between 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol and of cholesterol in response to Mg^{2+} and Ca^{2+} cannot be regarded as evidence for two 26-hydroxylase activities but may very well be related to the transport of the steroids to the enzyme. This assumption appears strengthened by the finding that there is a difference in effect of Mg^{2+} on the 26-hydroxylations of exogenous and endogenous cholesterol. It is apparent that a more definite answer requires studies with solubilized preparations. So far, attempts to solubilize mitochondrial 26-hydroxylase have failed.

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